REMARKS/ARGUMENTS

I. Status of the Claims

Claims 1-9, 11, 19-24, 52-55, 57, 59-65, 68-75, 77, 79-88, 90-99, and 101-103 are pending. Claims 8, 10, 12-18, 25-51, 56, 58, 66, 67, 76, 78, 89, 98, and 100 have been canceled. Claims 19-24, 59-65, 79-88, 90-97, 99, and 101-103 have been withdrawn from examination.

II. The Amendments Herein

The amendments herein add no new matter.

The amendments to the specification add information about patents and to clarify whether SEQ ID NO:1 is the sequence of the 3B3 antibody or of 3B3(Fv), as requested by the Examiner and correct typographical errors. Reference to an unnecessary European application is deleted, and information about a relevant Federal grant is added. These amendments were previously made in the amendment dated December 9, 2003, but not entered.

The claims have been amended to specify that the binding affinity and specificity of the claimed anti-gp120 antibodies are measured against the affinity and specificity of 3B3 Fv (SEQ ID NO:1). The recitation is supported throughout the specification, including page 2, lines 29-32.

Entry of the present amendments is respectfully requested as they do not introduce new issues for search and examination and either place the claims in condition for allowance or reduce the issues for appeal.

III. The Telephone Interview

The Applicants thank the Examiner for arranging and participating in the telephone interview held September 1, 2005 with Examiner Zeman, Supervisory Examiner Smith, and the undersigned counsel. Applicants believe that the interview was helpful in understanding the Examiner's position and thereby in expediting prosecution. As provided by MPEP §713.04, the substance of the points discussed in the interview are set forth below so that it is of record in the proceeding.

IV. The Office Action

The Final Office Action (hereafter, the "Action") rejects the claims on various grounds. Applicants amend in part and traverse the rejections. For the Examiner's convenience, the rejections are addressed below in the order in which they are presented in the Action.

A. Objections to the Specification

The Action maintains the objection to the specification for referring to U.S. patent applications that have since issued as patents. The amendments are believed to obviate the rejection. The Action further maintains the objection that it is not clear whether SEQ ID NO:1 is the sequence of the 3B3 antibody or of 3B3(Fv). The specification has been amended to clarify the references to SEQ ID NO:1.

B. Rejection for alleged lack of enablement

Claims 8, 10, 55, 56, 58, 74, 76, and 78 remain rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. According to the Action, the specification does not explicitly define what sequences and linkers comprise either 3B3(Fv) or 3B3(dsFv). The Action notes that the linker in SEQ ID NO:1 is an exemplar linker and states "The fact that the linker . . . is variable, there is no unique sequence associated with the identifier 3b3(Fv)." Action, at page 4. With respect to the dsFv, the Action states that "while the skilled artisan would be able to form disulfide-stabilized Fvs, there is no specific disclosure as to which amino acids in the 3B3 antibody need to be replaced with cysteines when forming the 3B3(dsFv). Consequently, the specific sequence associated either [with] 3b3(Fv) or 3B3(dsFv) is not known." Action, at page 5. The Action concludes that "[s]ince the specification provides no sequences for said material and one of skill would not be able to discern what VH and VL sequences of the 3B3 antibody are incorporated into the claimed 3B3(Fv) or 3B3(dsFv), deposit of the aforementioned biological material is required. Applicants amend in part and traverse.

In the telephone interview, the Examiner clarified that the concern was that the claims in question referred to particular molecular entities, but the exact sequence of the entity referenced was not specified. For the sake of good order, Applicants respectfully note that this rejection is based on an incorrect reading of the claims. The recitation of "3B3(Fv)-PE38" and "3B3(dsFv)-PE38" in the rejected claims could be met by any of a number of constructs. For example, the "3B3(dsFv)" portion of a "3B3(dsFv)-PE38" immunotoxin could reflect any antibody in which the VH and VL chains of the 3B3 Fv are joined by a disulfide bond between cysteine residues. Similarly, the "3B3(Fv)-PE38" recitation is satisfied by any immunotoxin in which the toxic moiety is targeted by the VH and VL chains set forth in SEQ ID NO:1, regardless of the particular linker joining the two chains.

Although the Applicants therefore maintain that the rejection is grounded on an incorrect reading of the claims, to expedite prosecution, the claims reciting "3B3(Fv)-PE38" or "3B3(dsFv)-PE38" have been cancelled. Applicants note for the record that the cancellation of these claims is not intended or believed to narrow the coverage of the claims, as the claims pending after the amendment are broader than those canceled. The cancellation of the claims to which the rejection pertains, of course, should obviate the rejection.

C. Rejection of the Claims as Obvious over Matsushita

Claims 1-6, 8, 9, 11, 52-55, 57, 68-72, and 74-77 are rejected under 35 U.S.C. § 103(A) as obvious over Matsushita et al., AIDS Research and Human Retroviruses, 6:193-203 (1990) ("Matsushita") in view of Barbas, Proc Natl Acad Sci 91:3809-3813 (1994) ("Barbas") and Pastan, U.S. Patent No. 5,458,878 ("Pastan"). According to the Action, Matsushita discloses the anti-gp120 antibody 0.5β coupled to PE. The Action states that, given that Matsushita discloses an antibody reactive with a number of HIV isolates, it would have been obvious for one of ordinary skill to use the 3B3 antibody in the Matsushita immunotoxin. Applicants traverse.

As Applicants noted in the amendment dated December 9, 2003, the rejection rests on the assumption that the person of skill in the art would have been motivated to modify

the Matsushita antibody based on the results set forth in the reference. Applicants observed that Matsushita was published in 1990 and that, following the publication of Matsushita, the only anti-HIV-1 immunotoxin to reach clinical trials (the immunotoxin was also directed against HIV-1 env) showed disappointing results. Applicants presented abstracts from Ramachandran et al., J. Infect Dis 170:1009-13 (1994) ("Ramachandran") and Davey et al., J. Infect Dis 170:1180-8 (1994) ("Davey") reporting the disappointing results. Applicants further pointed out that these results were referenced in the specification at page 35, line 26 to page 36, line 6, which observes that an immunotoxin using a portion of the CD4 molecule containing the gp120 binding site had unexpectedly high toxicity. Applicants also presented a copy of Goldstein et al., J. Infect Dis 181:921-926 (2000) ("Goldstein"), which states that, in the wake of these reports, the approach of using anti-HIV antibodies as targeting moieties for anti-HIV immunotoxins was abandoned. Applicants therefore maintained that the teachings in the art shortly after the publication of Matsushita were that use of anti-gp120 antibodies to target immunotoxins to HIV-infected cells would not work and that practitioners therefore had no motivation to modify Matushita as argued in the Action.

The present Action maintains the obviousness rejection. According to the Action, the "Ramachandran et al. reference is drawn to CD4-PE40 immunotoxins ware are not analogous to the instant invention. Hence any 'results' based on the [a]pplication of said immunotoxin would not have any bearing on the perceived efficacy of immunotoxin based on the combination of the cited references." Action, at page 7. Similarly, the Action states that the "Davey et al. reference is drawn to sCD4-PE40 immunotoxins which are not analogous to the instant invention. Hence any 'results' based on the [a]pplication of said immunotoxin would not have any bearing on the perceived efficacy of immunotoxin based on the combination of the cited references." *Id.* Finally, the Action states that the "Goldstein et al. reference was published in 2000, which was after the invention "was made" and hence would not have been part of the art at the time the invention was made." *Id.*

As an initial matter, Applicants respectfully observe that the Action does not explain or set forth any reasoning why considers the CD4-PE40 immunotoxin of the

Ramachandran reference and the sCD4-PE40 immunotoxin of the Davey reference are not analogous to the anti-gp120 immunotoxins of the present invention. The Action merely states it as a conclusion. The Applicants respectfully note that it is difficult for an applicant to respond to a rejection or, indeed, to determine whether or not it has been properly made, when the office action sets forth only a conclusion and not the reasoning on which the rejection is assertedly made. Applicants respectfully maintain that proper examination practice requires that the examiner set forth his or her reasoning, not just his or her conclusions.

In the telephone interview, the undersigned counsel pointed out that the MPEP states that a reference is analogous prior art for the purpose of analyzing obviousness of the subject matter at issue if it is either "in the field of applicant's endeavor or, if not, then [] reasonably pertinent to the particular problem with which the inventor was concerned." MPEP § 2141.01(a), *quoting In re Oetiker*, 24 USPQ2d 1443 (Fed. Cir. 1992). MPEP §2141.01(a) continues by stating that "A reference is reasonably pertinent if, even though it may be in a different field from that of the inventors's endeavor, it is one which, because of the matter with which it deals, logically would have commended itself to an inventor's attention in considering his problem." *Id., quoting In re Clay*, 23 USPQ2d 1058 (Fed. Cir. 1992) (emphasis added).

Since CD4-PE40 immunotoxins and gp120-PE immunotoxins are both immunotoxins intended to kill HIV-infected cells, the undersigned counsel observed during the interview that it was hard to understand on what basis Ramachandran and Davey would not "logically . . . have commended [themselves] to an inventor's attention in considering his problem," and therefore had to be considered analogous art under the standard set by the Court and adopted by the MPEP. The undersigned counsel further stated in the interview that the Examiner's point that the Goldstein reference was not part of the art at the time the invention was made was not well taken since it was cited not for something not known at the time of the invention, but for its retrospective statement as to what persons of skill thought following the publication of the Ramachandran and Davey studies. The undersigned pointed out that the Goldstein reference was therefore cited for what it stated about the art following the publication

of the Ramachandran and Davey studies, not for any developments that post-dated the filing date.

In the telephone interview, the Examiner stated that he would accept the references as analogous art, and clarified the basis of the rejection. The Examiner stated that he considered that immunotoxins directed to CD4 would bind and kill healthy T-helper cells (which express CD4), while the immunotoxins of the invention, which are targeted by antibodies to gp120, would bind to and kill only cells which display gp120, which is only present on cells infected with HIV. (As the Examiner knows, gp120 is a glycoprotein on the surface of HIV-1 that, in relevant part, binds CD4. Cells infected with HIV-1 express gp120 on their surface. See, e.g., specification, at page 34, lines 18-23.) As the Applicants understand the Examiner's contention, it is that the Ramachandran reference and the Davey reference did not remove the motivation provided by Matsushita, as argued by the Applicants, because one of skill would have concluded that the toxicity displayed by the CD4-PE40 and sCD4-PE40 immunotoxins would not have been exhibited by immunotoxins directed to gp-120-infected cells.

Upon reflection, Applicants believe the Examiner's position cannot be sustained. First, CD4-PE40 would be expected to bind only to cells that express the gp120 protein, which binds to CD4. Thus, there is no difference between the cells targeted by a CD4-targeted immunotoxin and an anti-gp120 antibody-targeted immunotoxin: they both target cells expressing gp120 on their surface.

Second, the problem CD4-targeted immunotoxins showed in clinical trials was not related to the loss of CD4+ T-cells or to loss of immune function, as might have been expected from targeting CD4-expressing cells. Rather, it was unexpected liver toxicity. See, specification at page 36, lines 3-12. This toxicity had not been seen during preclinical studies in animals at doses 25 times higher than the maximal dose humans proved able to tolerate. See, pages 35-6, bridging paragraph. If liver cells expressed the CD4 molecule, it might be expected that liver toxicity would have been seen in the pre-clinical studies. The absence of such toxicity in animals suggests that the toxicity seen in human trials was not due to some direct interaction between CD4 on liver cells, and therefore was not because the immunotoxin targeted healthy

cells along with HIV-infected cells, as suggested by the Examiner. Accordingly, the position suggested by the Examiner does not appear to withstand scrutiny.

Although the above comments are sufficient to rebut the Examiner's contention, for extra measure it is noted that, shortly after the priority date of the present application, Dr. Pastan and scientists from the National Institute of Allergy and Infectious Diseases ("NIAID"), published a "Perspective" in the Proceedings of the National Academy of Sciences. See, Berger, Moss and Pastan, Proc Natl Acad Sci 95:1151-11513 (1998) (copy enclosed. The abstract of this article was provided with the Applicants' December 2003 amendment). This article was edited by Dr. Anthony Fauci (see page 11511). (Dr. Fauci has been the Director of the NIAID since 1984. This fact, too, is immediately verifiable and not reasonably subject to dispute.) As the undersigned pointed out in the interview, the reference was prepared for the scientific literature, not for presentation to the Patent Office. The following is the statement read to the Examiner during the interview:

> The high hopes from the promising preclinical findings [with respect to the use of CD4-PE40 immunotoxin] were dashed in the initial Phase I trials with HIV-infected patients [citations omitted]. . . . The significant but reversible hepatoxicity greatly diminished enthusiasm for CD4-PE40 in particular and for Env-targeted toxins in general. The CD4-PE40 clinical program was terminated."

Thus, as pointed out in the previous response and during the interview, the failure of the CD4-PE40 immunotoxin in clinical trials was considered by those of considerable skill in the art to teach away not only from the use CD4-directed toxins, but also of env-targeted toxins in general, presumably including the use of gp-120-targeted toxins. Applicants appreciate that the Examiner has suggested a different conclusion might have been drawn but, with respect, the Examiner's conclusion was not the one the reference shows was drawn by persons in the art knowledgeable about immunotoxins in general and HIV-1 infections in particular.

The undersigned pointed out in the telephone interview that, although this article appeared after the priority date of the application, the statement in question is a characterization of the state of the art after the CD4-PE40 trials and prior to publication of the article. The

undersigned pointed out that this statement, and the corresponding statement in the Goldstein reference that the Action did not consider as evidence because it was after the priority date, are retrospective statements that do not introduce or rely on information that was not available prior to the priority date. Accordingly, the undersigned submits that they should be considered.

Applicants note that, under new rule 37 C.F.R. § 1.116(e), the consideration of additional evidence after a final action requires a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. Applicants respectfully note first, that the cited language from Berger et al. was cited by the undersigned during the interview, second, that the abstract of the reference is already of record, and third, that the reference is submitted in part in response to the Examiner's contentions during the interview, which were not explained or present in the final action. Accordingly, Applicants maintain that the submission of the reference at this time is appropriate under §1.116(e).

The Examiner is also respectfully reminded of the secondary considerations of obviousness set forth by the Court in *Graham v. John Deere*, 383 U.S. 1, 148 USPQ 459 (1966). MPEP 2141 makes clear that evidence of secondary considerations of non-obviousness is to be evaluated by the Examining Corps in every case. Among these secondary considerations is "long-felt need." As stated in the specification, at page 1, first paragraph under Background of the Invention, tremendous efforts have been made on understanding the pathogenesis of AIDS and in developing therapeutics. There is therefore considerable desire and a long felt need to develop additional therapeutics against HIV-1 infections. (Applicants assume that at this point, more than 20 years into the AIDS epidemic, it cannot reasonably be disputed that millions of persons worldwide are infected by HIV-1 or that there has been intense research into approaches to treating AIDS. Applicants respectfully suggest these are facts which, as required by MPEP 2144.03, are "capable of such instant and unquestionable demonstration as to defy dispute.")

Despite this long-felt need, PubMed searches for references on anti-gp120 immunotoxins and on anti-HIV immunotoxins show only a handful of publications pertaining to either of these matters between the publication of Matsushita in 1990 and the priority date of the present application in 1998. None of the references that came up on the search for anti-gp120

immunotoxins or on the anti-HIV immunotoxin search following publication of the Matsushita reference appear from their titles actually to relate to the making or testing of anti-gp120 immunotoxins. In fact, from the titles, the Matsushita reference is the only reference that came up in the search for anti-gp120 immunotoxin references that actually appears to relate to making an anti-gp120 immunotoxin. Copies of the two PubMed searches are enclosed. (For accuracy, a publication, Pincus and McClure, Proc Natl Acad Sci 90:332-336 (1993) was noted in which, although the title does not mention it, anti-gp120 immunotoxins were used. A copy of this publication is also enclosed. Interestingly, although anti-gp120 immunotoxins were used in the study, they appear to be of no interest to the authors as possible therapeutics.) Applicants maintain that this evidence is appropriate to submit at this time as the Goldstein reference submitted in response to the first office action was not considered by the Examiner on the basis that it post-dated the priority date. The present submission shows that the Goldstein reference's summarization of the state of the art is supported by the literature in the field.

Applicants respectfully submit that the fact that there does not appear to be a single publication on the development of an anti-gp120 immunotoxin in the approximately eight years between the publication of Matsushita and the priority date of the present application, despite the long felt need to develop anti-HIV therapeutics and the failure of CD4-PE40 immunotoxin, is clear evidence that persons of skill were not motivated by Matsushita, alone or in combination with Barbas and Pastan, to develop an anti-gp120 immunotoxin. Pursuant to MPEP § 2141, the failure of those in the art to develop a gp120-targeted immunotoxin despite the long felt need for HIV-1 therapeutics, and despite the failure of the CD4-PE40 immunotoxin, are secondary considerations of non-obviousness that must be taken into account and given appropriate weight by the Examiner.

In short, the Examiner's hypothesis presented during the interview does not withstand scrutiny, the Goldstein and Berger references show that any motivation Matsushita provided to create an anti-gp120 immunotoxin was ended by the results reported by Ramanchandran and Davey, and there are clear secondary considerations of non-obviousness.

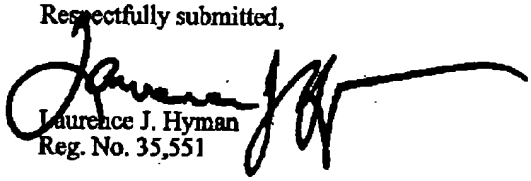
Reconsideration and withdrawal of the rejection in view of the discussion above is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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Attachments: Berger et al. Article
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Mol Med. 1998 Jun;4(6):384-91.
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Rationale for the use of immunotoxins in the treatment of HIV-infected humans.
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Items 21 - 26 of 26 Previous Page 2 of 2

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Proc. Natl. Acad. Sci. USA
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Perspective

Reconsidering targeted toxins to eliminate HIV infection: You gotta have HAART

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ABSTRACT **The success of highly active anti-retroviral therapy (HAART) has inspired new concepts for eliminating HIV from infected individuals. A major obstacle is the persistence of long-lived reservoirs of latently infected cells that might become activated at some time after cessation of therapy. We propose that, in the context of treatment strategies to deliberately activate and eliminate these reservoirs, hybrid toxins targeted to kill HIV-infected cells be reconsidered in combination with HAART. Such combinations might also prove valuable in protocols aimed at preventing mother-to-child transmission and establishment of infection immediately after exposure to HIV. We suggest experimental approaches *in vitro* and in animal models to test various issues related to safety and efficacy of this concept.**

Highly active anti-retroviral therapy (HAART), involving combination treatment with drugs that block different steps in the viral replication cycle (e.g., reverse transcriptase inhibitors plus protease inhibitors), has improved dramatically the health of many individuals infected with HIV (1). Despite these advances, recent analyses of peripheral blood and lymph nodes have revealed the presence of reservoirs of resting $CD4^+$ memory T cells harboring latent replication-competent provirus (refs. 2–8; reviewed in refs. 9–11). Although such reservoirs contain exceedingly small numbers of cells, they are generated very early after primary infection and persist with no significant change after 2 years of HAART. The latently infected cells are likely to activate spontaneously at some point after termination of HAART and therefore are considered to be a major obstacle to eradication of HIV from the body. This awareness has engendered the notion of deliberately "flushing out" the reservoirs by treating HAART patients with agents that activate virus expression from latently infected cells (10–12). The idea is that the virions produced on activation will be prevented by HAART from infecting new cells; it is presumed that the newly activated cells then will be eliminated by natural mechanisms such as the cytopathic effect of the virus, immune effector mechanisms, etc. (9–11). We propose that, in considering such strategies, these natural elimination mechanisms can be accelerated aggressively by using targeted toxins that selectively kill activated HIV-infected cells. Such agents may also be useful components of cocktails aimed at preventing establishment of infection in newly exposed individuals.

Hybrid Toxins Targeted to HIV-Infected Cells

During the past decade, several types of anti-HIV hybrid protein toxins have been produced by molecular genetic and biochemical methodologies (13–15). In each case, the hybrid protein contains a binding domain that targets the agent to the HIV envelope glycoprotein (Env) expressed on the surface of the infected cell and a cytotoxic domain that actively kills the cell on internalization. The hybrid toxins are constructed by substituting the normal cell binding region of the native toxin with an Env-binding domain. The Env-binding moieties used have included the extracellular regions of CD4 as well as Fab regions of anti-Env antibodies (directed against either the external subunit gp120 or the transmembrane subunit gp41). The cytotoxic domains have been derived from natural protein toxins such as *Pseudomonas aeruginosa* exotoxin A (PE), ricin, and diphtheria toxin.

To date, only one of these hybrid toxins has been tested in humans: the genetically engineered single chain protein CD4-PE40 (soluble CD4 linked to the translocation and cell killing domains of PE). For this reason, we focus on this agent, though many of our arguments also apply to other Env-targeted hybrid toxins. CD4-PE40 displays the following properties *in vitro*: cytotoxic activity against cells expressing Envs of HIV-1, HIV-2 and simian immunodeficiency virus (SIV) (16–19), high potency and specificity for killing HIV-1-infected cells with negligible effects on major histocompatibility complex Class II-expressing cells (16, 18), requirement for HIV-1 induction in a latently infected cell line (18), suppression of spreading HIV-1 infection in an acutely infected T cell line (17) and in cultures of primary T lymphocytes or macrophages (20–22), highly synergistic activity with reverse transcriptase inhibitors (23), and potent activity against primary HIV-1 strains, including those resistant to neutralization by soluble CD4 (21, 22). These *in vitro* properties, coupled with acceptable toxicity and pharmacokinetic profiles in animal studies, supported testing this agent in HIV-infected people.

Disappointing Results in Phase 1 Clinical Trials

The high hopes from the promising preclinical findings were dashed in the initial Phase I trials with HIV-infected patients (24, 25). The toxin produced dose-limiting hepatotoxicity; at the low doses that were tolerated (10 μg/kg), the peak plasma levels of CD4-PE40 remained below concentrations shown to be efficacious *in vitro*. The significant but reversible hepatotoxicity greatly diminished enthusiasm for CD4-PE40 in par-

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Abbreviations: HAART, highly active anti-retroviral therapy; Env, HIV envelope glycoprotein; PE, *Pseudomonas aeruginosa* exotoxin A; SIV, simian immunodeficiency virus; CD4-PE40, soluble CD4 linked to the effector domains of *Pseudomonas aeruginosa* exotoxin A.

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ticular and for Env-targeted toxins in general. The CD4-PE40 clinical program was terminated.

A New Context Suggests a New Concept: Testable Hypotheses

We propose that recent developments with HAART present new opportunities for exploring the therapeutic utility of Env-targeted hybrid toxins to help eradicate residual HIV-infected cell reservoirs. We hypothesize a plausible mechanism for the CD4-PE40 hepatotoxicity observed in HIV-infected people and suggest that this problem may not occur in patients with the very low viral loads achieved by HAART. We present rationales based on *in vitro* data suggesting that Env-targeted toxins might accelerate the elimination of infected cell reservoirs beyond the rates caused by natural mechanisms. These agents may also be useful components of drug cocktails aimed at preventing postexposure infection and mother-to-child transmission. Of most importance, many of these notions are subject to experimental testing *in vitro* and in animal models.

The first issue concerns the hepatotoxicity with low doses of CD4-PE40 observed in Phase I clinical trials. This problem was unexpected because preclinical toxicity studies in rodents and monkeys indicated much higher tolerated doses. Furthermore, it is now clear that hepatotoxicity is not a general property of PE derivatives in humans; several anticancer clinical trials conducted with PE-based immunotoxins have revealed striking antitumor responses without hepatotoxicity (26). Why, then, was hepatotoxicity encountered with low doses of CD4-PE40 in clinical trials with HIV-infected people? We propose that the HIV infection, particularly the high virus load, is the culprit. HIV-infected individuals likely produce free gp120 that is shed from virions or infected cells. Shedding of gp120 has been studied extensively *in vitro*; furthermore, evidence for free gp120 in sera of infected individuals has been reported, although precise measurements are confounded by the formation of immune complexes as well as by the association of released gp120 with circulating $CD4^+$ T lymphocytes (reviewed in refs. 27–29). We hypothesize that, in HIV-infected patients treated with CD4-PE40, some of the chimeric toxin associates with shed gp120. Because gp120 is glycosylated extensively and contains highly diverse oligosaccharide chains (30), the complex likely would be a substrate for the human hepatocyte asialoglycoprotein receptor, which internalizes glycoproteins containing terminal galactose or N-acetylglucosamine residues (31). The result would be the serious side effect of hepatocyte killing. Moreover, gp120/CD4-PE40 complexes bound to anti-gp120 antibodies might also contribute to liver damage.

According to this hypothesis, hepatotoxicity should not be a major problem in HAART patients because the low viral loads presumably would produce minimal amounts of free gp120. Even on induction of virus expression from latently infected memory T lymphocytes, the newly produced free gp120 is unlikely to be problematic because the number of such cells is much smaller than the number of virus-producing T lymphocytes in patients before HAART (ref. 3; also T.-W. Chun, personal communication); moreover, the newly produced gp120 will have accumulated only during the relatively short period after induction, in contrast with the prolonged duration of gp120 production before therapy. We also suggest that gp120-mediated toxicity would not be problematic when given along with HAART to newly exposed individuals because they should not yet have produced significant amounts of free gp120.

To test this model of CD4-PE40-mediated hepatotoxicity, we propose that effects of the agent be compared in animals with high vs. low levels of free gp120; we predict that hepatotoxicity will be much less severe in the latter case. There are several experimental paradigms in which this question can be examined, including comparison of CD4-PE40 hepatotoxicity in uninfected vs. chronically HIV-infected severe combined immunodeficient-hu mice or SIV-infected rhesus macaques. Perhaps more important is to use these systems to compare animals with the normal high viral loads occurring during chronic infection vs. the reduced loads achieved with potent antiviral therapy, e.g., HAART in HIV-infected severe combined immunodeficient-hu mice (32) or reverse transcriptase inhibitor therapy in SIV-infected macaques (33). A related analysis would compare in chronically infected animals the effects of hybrid toxins targeted to gp120 (e.g., CD4-PE40 and gp120-targeted immunotoxins) versus those targeted to gp41; according to our model, the latter agents would not produce hepatotoxicity even in animals with high virus load because gp41 is not released spontaneously from the membrane. In another approach, uninfected animals can be given CD4-PE40 without or with soluble gp120 to test directly whether hepatotoxicity depends on both proteins. Together, these experiments should provide important insights into whether the hepatotoxicity of CD4-PE40 is associated with high viral load, and in particular with free gp120.

HAART therapy also provides opportunities to test the therapeutic potential of Env-targeted hybrid toxins to eradicate residual infected cells. The idea is to augment their natural rates of decay, which are presumed to reflect the viral cytopathic effect and host effector mechanisms (9–11). Several previous *in vitro* studies are promising in this regard. CD4-PE40 (but not soluble CD4) markedly inhibited the spread of infection in various target cell types (17, 20–23), including primary T lymphocytes and macrophages acutely infected with primary HIV-1 strains; the interpretation of these findings is that the toxin accelerates the killing of infected cells beyond the rates associated with the viral cytopathic effect. The results with macrophages are particularly striking because these cells are refractory to HIV-mediated killing during productive infection and are thought to represent an important viral reservoir with markedly slower decay kinetics compared with $CD4^+$ T lymphocytes (9). Also of note are the promising *in vitro* results indicating highly synergistic effects of CD4-PE40 and reverse transcriptase inhibitors (23). CD4-PE40 plus 3′-azido-3′-dideoxythymidine or 2′,3′-dideoxyinosine completely inhibited acute virus replication and prevented virus-mediated killing of the $CD4^+$ target T cell population; moreover, continuation of the culture after cessation of drug treatment indicated that the infection had been eliminated completely. By contrast, each agent alone suppressed virus replication during the treatment period, but the protective effects were reversed on drug removal. These results highlight the potential value of combination treatment involving a drug(s) that inhibits HIV replication plus another that selectively kills the infected cells. Taken together, these earlier studies provide impetus for considering Env-targeted toxins to augment HAART, particularly in the context of protocols to deliberately activate virus production from latently infected cell reservoirs. These agents, in combination with other antiretrovirals, may also diminish the frequency of postexposure infection and mother-to-child transmission.

We propose additional lines of *in vitro* and *in vivo* study. Experiments can be designed to optimize the *ex vivo* activation of latently infected T lymphocytes obtained from HAART patients and to test *ex vivo* whether an Env-targeted toxin in combination with continued HAART promotes or accelerates killing of the activated cells (similar to the studies noted above with acutely infected T cells). As an *in vivo* parallel to the previous *in vitro* success with combination treatment, we propose examination of the combined effects of HAART and Env-targeted toxins in HIV-infected severe combined immunodeficient-hu mice or SIV-infected macaques. Regarding efforts to deliberately activate latently infected cells, *in vitro* experiments would guide the choice of the most promising

modes of activation. Such experiments would suggest whether Env-targeted toxins in the presence of HAART can eradicate virus from infected animals. Finally, the ability of the targeted toxins to augment other antiretrovirals in preventing infection can be examined in the HIV/severe combined immunodeficient-hu mouse and the SIV/macaque models. Favorable results in these *in vitro* and *in vivo* systems would set the stage for safety and efficacy trials of Env-targeted toxins as components of therapeutic and prophylactic protocols against HIV.

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Medical Sciences

Soluble CD4 enhances the efficacy of immunotoxins directed against gp41 of the human immunodeficiency virus

(combination therapy/AIDS/antibody/immunoglobulin)

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ABSTRACT Monoclonal antibodies specific for the gp120 or gp41 portions of the human immunodeficiency virus (HIV) envelope protein gp160 were conjugated to ricin A chain, and their immunotoxic activities against HIV-infected cells were evaluated in the presence or absence of soluble CD4 (sCD4). Immunotoxin activity was measured *in vitro* as cytotoxicity and inhibition of secretion of infectious HIV. The efficacy of anti-gp41 immunotoxins was enhanced at least 30-fold in the presence of sCD4. This effect was specific for HIV-infected cells, but not for uninfected cells, and was seen at concentrations of sCD4 as low as 0.1 μg/ml. Anti-gp120 immunotoxins were marginally inhibited at higher concentrations of sCD4. Flow cytometry analyses showed that sCD4 increased the expression of gp41 on the surface of infected cells and increased internalization of gp120 and gp41. These data suggest that sCD4 alters the cellular trafficking of HIV envelope proteins. These findings also have important implications for the therapeutic use of anti-HIV immunotoxins and may be generalizable to other immunotoxins as well.

CD4 is an integral membrane glycoprotein of helper T lymphocytes and serves as the cellular receptor for human immunodeficiency virus (HIV) (1). CD4 is bound by the extracellular portion of the viral envelope protein gp120. Upon binding to CD4 the envelope protein undergoes conformational changes, including release of free gp120 from virions (2, 3), loss of the envelope spikes as viewed by electron microscopy (4), and increased exposure of epitopes on the NH_2 terminus of the transmembrane portion of the envelope protein gp41 (5). These conformational changes are probably the earliest steps in the infectious process following the binding of HIV to its cellular receptor. They may also represent potential opportunities for therapeutic intervention.

One approach to the treatment of HIV infection is the elimination of cells that actively produce the virus. To this end, agents have been developed that use either antibodies or CD4 to deliver a toxic moiety to cells expressing the HIV envelope at the cell surface (6–11). These must bind and be internalized to kill the target cell. Accordingly, these reagents are not only potential therapeutic agents but also probes that may be used to study the cellular trafficking of retroviral proteins.

We have produced a panel of immunotoxins that consists of anti-envelope antibodies directed against different epitopes on gp120 and gp41 that are coupled to ricin A chain (RAC) (7). Because the addition of soluble CD4 (sCD4) has been shown to alter the conformation of the HIV envelope protein, the effect of sCD4 on the efficacy of anti-gp160 immunotoxins was tested. It is demonstrated that the *in vitro* efficacy of anti-gp41 immunotoxins was enhanced by at least 30-fold in the presence of sCD4. This effect is most likely due to an increase in the expression of gp41 epitopes on the surface of target cells as well as to increased rates of internalization of gp41.

MATERIALS AND METHODS

Antibodies, Immunotoxins, and sCD4. Specificities of monoclonal antibodies used in this study are listed in Table 1. Antibodies 41.1 and 41.4 bind to the immunodominant region of gp41, ~60 amino acids toward the COOH terminus from the fusion domain. Antibodies were coupled to RAC at a molecular ratio of 1:1 to produce immunotoxins as described elsewhere (7). CD4-PE40 (9, 11) was obtained from Upjohn, as was $sCD4_{183}$, which consists of the NH_2-terminal two domains of CD4 (16).

Cellular Assays. The following cell lines were used in these studies: H9 $CD4^+$ T-cell leukemia H9 (provided by M. Reitz, National Cancer Institute, Bethesda, MD); H9 cells persistently infected with the infectious molecular clone of HIV, NL4-3 (17, 18); and H9 cells infected with the HIV isolate $HTLV\text{-}III_{MN}$ (AIDS Research and Reference Reagent Program, Rockville, MD). Virtually 100% of H9/NL4-3 cells were actively producing HIV, whereas <1% of H9/MN secreted virus. $CD4^+$ HeLa cell line 1022 was used to detect infectious HIV (19).

Immunotoxin efficacy was monitored by two previously described assays (7). The cytotoxic effect of immunotoxins was measured on H9/NL4-3 cells in a 72-hr assay. Cells (3×10^4) were plated in 0.2 ml of RPMI 1640 medium with 10% fetal calf serum in flat-bottom 7-mm microtiter wells (Flow Laboratories). Protein synthesis was measured during the final 16 hr of culture as [^{35}S]methionine (0.5 μCi per well; 1 Ci = 37 GBq) incorporation, and the results are reported as counts per minute (cpm). The ability of the immunotoxin to inhibit the production of infectious HIV was determined using a specific focal immunoassay (FIA) (7, 19, 20). Monolayers of $CD4^+$ HeLa cells were plated at 3×10^4 cells per 2-ml (16-mm) well. HIV-infected cells were treated for 24–48 hr with the immunotoxin and/or sCD4. The cells were then plated in serial dilutions (3×10^5 to 100 cells per well, the range depending upon the particular cells being tested) on the monolayer of $CD4^+$ HeLa cells. Twenty-four hours later the cells were washed off of the monolayer. Foci of HIV-infected cells were detected by immunoperoxidase staining 2 days later. The results are reported as the percent of input cells that scored as infectious centers.

Flow cytometry was performed on H9/NL4-3 cells on a Becton Dickinson Facstar using the indicated monoclonal antibody and a species-specific fluorescein-conjugated anti-immunoglobulin secondary antibody as described elsewhere (7). Antibodies were used at a concentration of 10 μg/ml,

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Abbreviations: HIV, human immunodeficiency virus; FIA, focal immunoassay; RAC, ricin A chain; sCD4, soluble CD4.

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Table 1. Monoclonal antibodies used in this study

mAb	Species	Specificity	Reactivity with HIV isolates	Source or ref.
T7	Mouse	Irrelevant antigen	None	12
924	Mouse	gp120, V3 loop amino acids 313–324	IIIB only	7
F58	Mouse	gp120, V3 loop amino acids 309–317	Broadly reactive	13
10B	Humanized*	Irrelevant antigen	None	14
41.1	Human	gp41, amino acids 579–604	Broadly reactive	J.M.
41.4	Human	gp41, amino acids 579–604†	Broadly reactive	J.M.

Specificity of monoclonal antibodies (mAbs) was determined on synthetic peptides; amino acid numbering conforms to HXB-2 envelope sequence (15).
*Antibody 10B is a chimeric antibody with mouse variable regions and human constant regions.
†Antibody 41.4 reacts weakly with a peptide representing these amino acids, binds to recombinant gp160 with higher affinity than 41.1, and cross-competes with 41.1. Thus 41.4 most likely binds to a conformational epitope in this region.

determined to be a saturating concentration by prior titration. Cells were incubated sequentially with each antibody for 1–2 hr at room temperature in the presence of 0.1% sodium azide. To determine rates of internalization of HIV antigens, monoclonal antibodies were directly conjugated with 6-(fluorescein-5-carboxamido)hexanoic acid, succinimydal ester (Molecular Probes) according to the manufacturer's protocol at a fluorescein-to-protein ratio of 7:1. Cells (10^6) were incubated with sCD4 (1 μg/ml) for 2 hr at 37°C and then placed in the presence of the directly fluoresceinated antibody (30 μg/ml) at 37°C in RPMI 1640 medium containing 10% fetal calf serum. At the indicated time they were transferred to cold medium containing 0.5% sodium azide. Fluoresceinated antibody detecting cell surface antigen was quenched by incubation of the cells for 1 hr at 4°C in anti-fluorescein antibody (Molecular Probes) at a concentration previously determined to be in 3-fold excess for inhibiting cell surface fluorescence. Total fluorescence (unquenched) and internal fluorescence (quenched) were determined by flow cytometry. Calculations were based upon the mean fluorescent value for the entire cell population studied (i.e., no fluorescence gates were set). Internal fluorescence at each time point was calculated by subtracting the quenched fluorescence at time zero (i.e., the background fluorescence before any internalization could occur) from the quenched fluorescence at the indicated time.

RESULTS AND DISCUSSION

Because the addition of sCD4 has been shown to alter the conformation of the HIV envelope protein, the effect of sCD4 on the efficacy of anti-gp160 immunotoxins was examined. To determine if there was enhancement of immunotoxin efficacy, immunotoxins were tested at different concentrations in the presence or absence of 1 μg of sCD4 per ml (Fig. 1). Immunotoxin efficacy was measured as inhibition of protein synthesis in persistently infected H9/NL4-3 cells. Two different immunotoxins were tested: 41.1-RAC and 41.4-RAC. CD4-PE40 was included as a control. In the absence of sCD4, CD4-PE40 and 41.4-RAC were equally efficacious, whereas 41.1-RAC gave equivalent inhibitions at a third of the dose of the others. When sCD4 was added to CD4-PE40, the expected inhibition of its action was observed. The activities of 41.4-RAC and 41.1-RAC were significantly enhanced by sCD4. Equivalent toxicity by 41.4-RAC was seen at 0.01 μg/ml in the presence of sCD4 and 0.3 mg/ml in its absence, indicating a 30-fold enhancement of immunotoxin efficacy. With 41.1-RAC, complete suppression of protein synthesis was seen in the presence of sCD4 at the lowest dose tested (0.01 μg/ml), indicating that the enhancement was at least 30-fold, possibly greater. A similar dose–response was performed on uninfected H9 cells at immunotoxin concentrations as high as 100 μg/ml (data not shown). There was no suppression of protein synthesis in uninfected cells by either 41.1 or 41.4-RAC in the presence or absence of sCD4 at any immunotoxin concentration. In contrast, nonspecific toxicity of CD4-PE40 on uninfected H9 cells was seen at concentrations >30 μg/ml.

The concentration of sCD4 required to produce an effect on immunotoxins was next determined (Fig. 2). Anti-gp41 immunotoxins (41.1 and 41.4-RAC) were tested at the suboptimal concentration of 0.08 μg/ml. Maximal enhancement of their effect could be observed at concentrations of sCD4 as low as 0.1 μg/ml. sCD4 has been reported to produce the release of free gp120 from HIV virions and infected cells and to decrease the exposure of gp120 antigens on the surface of infected cells (3, 5). We therefore tested anti-gp120 immunotoxins F58 and 924-RAC at effective doses (2 μg/ml) and looked for diminution of their effect by sCD4. At concentrations of sCD4 >1 μg/ml, a modest decrease in the efficacy of the 924, but not F58, immunotoxin was noted (Fig. 2).

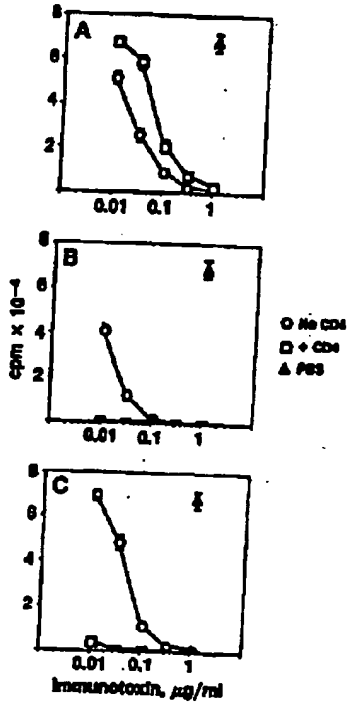

FIG. 1. Dose–response of immunotoxins in the presence or absence of sCD4. The ability of three different immunotoxins [CD4-PE40 (A), 41.1-RAC (B), 41.4-RAC (C)] to inhibit protein synthesis in H9/NL4-3 cells was determined in the presence or absence of sCD4 (1 μg/ml). Immunotoxins were tested over a 100-fold range of concentrations. PBS indicates protein synthesis in the absence of any immunotoxin.

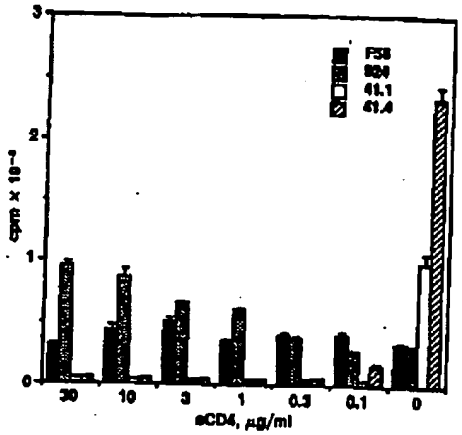

FIG. 2. Dose–response of sCD4 effect upon immunotoxins. The effect of anti-gp41 and anti-gp120 immunotoxins was measured on H9/NL4-3 cells in the presence of various concentrations of sCD4. Anti-gp41 immunotoxins 41.1-RAC and 41.4-RAC were tested at a suboptimal concentration (0.08 μg/ml), whereas anti-gp120 immunotoxins 924-RAC and F58-RAC were tested at an efficacious dose (2 μg/ml). [^{35}S]Methionine incorporation in H9/NL4-3 cells in the absence of immunotoxin was 84,889 cpm.

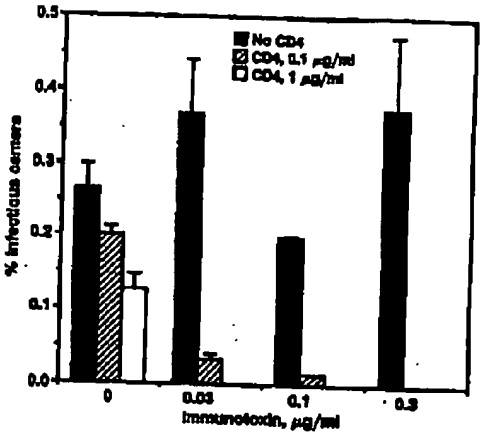

FIG. 3. Inhibition of production of infectious HIV by combinations of immunotoxin and sCD4. H9 cells infected with the MN strain of HIV were incubated for 48 hr in the presence of the indicated concentrations of sCD4 and 41.4-RAC. Serial dilutions of the cells (3 × 10^2 to 10^3 cells per well) were then transferred to monolayers of CD4$^+$ HeLa cell line 1022. Three days later, the number of foci were enumerated by immunoperoxidase.

The nature of the conformational change induced by sCD4 has not been precisely defined. To probe this, monoclonal antibodies directed against regions of gp160 that are exposed on the soluble envelope protein, but not functionally accessible on cell-associated gp160, were used, hoping that sCD4-induced conformational changes may expose these cryptic epitopes on the cell surface. The following monoclonal antibodies (with their epitopes indicated) were coupled to RAC: H11 (amino acids 472–477), C6 (amino acids 91–99), B13 (amino acids 256–264), 110.1 (amino acids 200–217), C-1 (amino acids 492–498), and C8 (amino acids 727–732). All but the last recognize epitopes in gp120. When tested in the presence or absence of sCD4 these immunotoxins had no effect on H9/NL4-3 cells (data not shown). Thus, sCD4-induced conformational changes do not include the exposure of these epitopes on the cell surface.

To demonstrate that the effect of sCD4 on immunotoxin action was not restricted to H9/NL4-3 cells, a different HIV isolate and different cell types were tested. The FIA was used to assess the efficacy because in these situations only a small proportion of the cells was expressing viral antigens, rendering the measurement of cytotoxicity on these populations meaningless. The combined effect of 41.4-RAC and sCD4 on H9 cells infected with the HIV isolate MN is shown in Fig. 3. The immunotoxin alone was ineffective at all three doses tested. sCD4 alone showed a small, but significant, dose-related neutralization of the infection of the indicator CD4$^+$ HeLa cells by the MN virus. However, when immunotoxin and sCD4 were used in conjunction, virtually complete inhibition of HIV infectivity was seen at all doses tested. A similar experiment was performed using CEM and phytohemagglutinin (PHA)-stimulated blast cells acutely infected with NL4-3 strain. With the CEM cells, the addition of sCD4 enhanced the efficacy of the 41.4 immunotoxin at least 10-fold (testing was not done for greater increases). Additive efficacy of sCD4 and 41.4-RAC was also seen on the PHA blasts (not shown).

The mechanism of sCD4 enhancement was studied using flow cytometry. In the first experiment, indirect immunofluorescence was used to assess the increase in cell surface antigen expression following treatment of H9/NL4-3 cells with sCD4 (10 μg/ml) at 37°C (Fig. 4*A*). The data indicated that there was an increase in exposure of gp41 epitopes and a decrease in gp120. This effect was seen at lower doses of sCD4 as well and was consistent with previously published work (5). However, additional factors may also account for the enhancement of immunotoxin efficacy. Since internalization of immunotoxins is important for their function, the effect of sCD4 on internalization of antibodies bound to envelope antigens was examined. In this experiment directly fluoresceinated antibodies were incubated with H9/NL4-3 cells at 37°C for increasing periods of time. Cell metabolism was then paralyzed with sodium azide. The fluorescence of antibody bound to cell surface antigens was quenched with anti-fluorescein antibody. Internalized antibody was then detected by flow cytometry (Fig. 4*B*). sCD4 (1 μg/ml) enhanced the internalization of antibodies 924 (anti-gp120) and 41.4 (anti-gp41) but not the irrelevant control antibody 10B. This effect was not due to increased binding of the antibody to sCD4-treated cells, since it was seen with antibody 924, whose binding is decreased by sCD4.

These data indicate that in addition to the previously described effects of sCD4 on the conformation of the envelope protein, sCD4 also affects the intracellular circulation of the HIV envelope protein(s) in infected cells. Increased internalization, taken together with the alterations in cell surface antigen exposure, can explain the effects of sCD4 on anti-gp41 and anti-gp120 immunotoxins. Enhancement of anti-gp41 immunotoxin activity is a result of increased exposure of gp41 epitopes on the cell surface and increased internalization. sCD4 has little effect on the efficacy of anti-gp120 immunotoxins because decreased antigen exposure is offset by increased rates of internalization.

Under the experimental conditions used here, there was a synergistic interaction between sCD4 and anti-gp41 immunotoxins. The efficacy of these immunotoxins was enhanced at least 30-fold by the addition of sCD4. There was no indication that the enhancement was accompanied by any increase in nonspecific toxicity. CD4-PE40 also acts by binding to HIV envelope protein on the surface of infected cells, is internalized, and then kills the cells. The data shown here demonstrate that CD4-PE40 and the anti-gp41 immunotoxins 41.1-RAC and 41.4-RAC have very similar *in vitro*

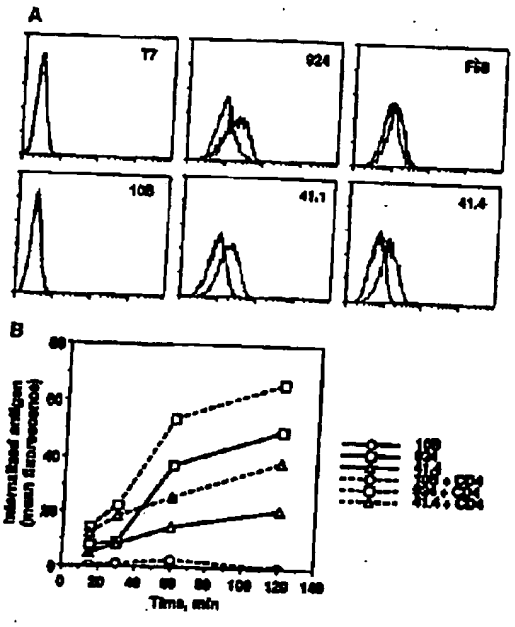

FIG. 4. Flow cytometric analyses of sCD4 effects on H9/NL4-3 cells. (*A*) Indirect immunofluorescent analyses of antigen exposure following incubation in the presence (broken line) or absence (solid line) of sCD4 (10 μg/ml) for 2 hr at 37°C are shown. Antibodies T7 and 10B are irrelevant control antibodies, 924 and F58 are anti-gp120, and 41.1 and 41.4 are anti-gp41. Cell number is shown on the ordinate; log fluorescence intensity is shown on the abscissa. (*B*) The effect of sCD4 on the internalization of envelope antigens was measured by flow cytometry. Cells were incubated with directly fluoresceinated antibodies for the indicated time at 37°C and then poisoned with sodium azide. Cell surface fluorescence was quenched with an anti-fluorescein antibody. Internalized antigen was then detected by flow cytometry. Changes in cell surface antigen expression (mean fluorescence) induced by sCD4 were a decrease of 46 fluorescence units for antibody 924 and an increase of 111 for antibody 41.4 (at the 30-min time point).

dose–response curves on persistently infected cells in the absence of sCD4; but when sCD4 is added the RAC-conjugated monoclonal antibodies are considerably more potent (Fig. 1). Further advantages of monoclonal antibody-based immunotoxins include a longer serum half-life (21, 22) and less nonspecific toxicity on uninfected tissue culture cells (see above). However, it should be noted that CD4-PE40 may be somewhat more active against acutely infected cells than the anti-gp41 immunotoxins (S.H.P., unpublished), most likely due to the additive effect of neutralization and toxin activity that we have previously described (7). If immunotoxins are shown to have clinical utility, it is likely that treatment will involve panels of different immunotoxins because of immunogenicity and HIV variation (23). There will be a place for immunotoxins based on monoclonal antibodies and CD4 toxins in the treatment of AIDS.

We have shown *in vitro* enhancement of immunotoxin action by sCD4 but at this time can only speculate about whether this effect will be true *in vivo* as well. It is clear that the concentrations of immunotoxin and sCD4 at which we observe *in vitro* effects are readily attainable *in vivo* (24–26). Moreover, with the introduction of CD4-immunoglobulin derivatives, the serum half-lives of CD4 and immunotoxins are almost identical (21, 22, 24). Thus, it should be possible to coadminister the immunotoxin and CD4-immunoglobulin *in vivo* and obtain the effects we have reported here.

The exact nature of the conformational change induced by the binding of CD4 to the gp120/gp41 complex is still a matter of conjecture (5, 27, 28). There are likely multiple points of attachment between CD4 and gp120 (28). Following this binding, gp120 dissociates from gp41 to a variable degree (2, 27). There is a concomitant increase in the exposure of the NH_2 terminus of gp41 (5), which contains the fusion domain. This in turn allows the next step in viral entry, membrane fusion, to proceed. The increase in exposure of the NH_2 terminus of gp41 may result from any or all of the following: (*i*) conformational changes in the gp120/gp41 complex, (*ii*) changes in the state of gp41 oligomerization, (*iii*) dissociation of gp120 from gp41 (5). Two pieces of data reported here bear on these issues. The first is the failure of sCD4 to cause exposure of cryptic epitopes on either gp120 or gp41, suggesting that there are not global conformational changes induced in envelope protein by CD4 binding. Second, we demonstrated that CD4 binding enhances the internalization of gp120 and gp41. Similar findings are observed when cell surface receptors bind their ligands and are internalized via coated pits, a phenomenon associated with increased oligomerization of the receptor. By analogy, our data suggest that CD4 binding may alter the state of gp120/gp41 oligomerization.

A caveat on the utility of the results described here is that patient-derived HIV may be less sensitive to sCD4-mediated neutralization than are laboratory strains (27). It may also be true that patient-derived isolates are less susceptible to sCD4-mediated enhancement of anti-gp41 immunotoxin action and, for that matter, to the toxic effects of CD4-PE40.

The enhancement of immunotoxin activity described here may be generally applicable to other immunotoxins. gp160/sCD4 may be considered analogous to other receptor/ligand systems. A number of immunotoxins directed against cellular receptors for growth factors have been proposed for the therapy of cancer. Examples include transferrin, epidermal growth factor, platelet-derived growth factor, and interleukin 2. Increased internalization of many receptors in the presence of their ligands has been demonstrated. Thus the internalization of anti-receptor immunotoxins, and consequently their therapeutic efficacy, may be enhanced by the addition of free ligand. Such an observation would greatly increase the utility of immunotoxins in the therapy of cancer as well as AIDS.

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